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Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits

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Summary

Storage of swine manure is associated with the microbiological production of a variety of odorous chemicals including ammonia, organic acids and alcohols, and sulphides. Although largely the product of microbiological activity, little is known about the microorganisms present in swine manure. In order to gain a better understanding of the types and activities of the microorganisms present, representative strains of microorganisms were isolated from faeces and stored manure slurry, identified, and physiologically characterized. For swine manure slurry samples, total anaerobe colony counts were greatest when a nonselective, habitat simulating medium containing clarified swine manure slurry was used whereas the highest counts for faecal anaerobes were obtained on rumen fluid containing medium. Faecal and slurry samples were also plated onto the appropriate medium containing the antibiotics tetracycline, erythromycin and tylosin (10 μg ml^{-1}, individually) and the proportional counts of organisms capable of growing in the presence of these antibiotics determined. Randomly selected isolates from the highest dilutions were identified by 16 s rDNA sequence analysis, and selected physiological characteristics were determined. The results of these examinations indicate that the predominant culturable microorganisms from these environments are obligately anaerobic, low mol percentage G + C Gram positive bacteria (Firmicutes) who are members of Clostridial, Eubacterial, and Lactobacillus/Streptococcus phylogenetic groups. Isolates similar to Sporomusa and Flexibacter/Cytophaga/Bacteroides (CFB or Bacteroidetes) groups were also obtained. Although similar overall, faecal and slurry samples differed in bacterial composition. Manure slurry samples were dominated by organisms similar to *Clostridium coccoides* and *Enterococcus* species whereas the distribution of species present in faeces appeared much broader. Whereas most of the pure cultures could be assigned to known phylogenetic groupings, few could be identified as known species. Examination of some growth and physiological characteristics of faecal and slurry isolates showed these to be primarily carbohydrate fermenters, although some were able to ferment lactate and amino acids. When the ability of manure and faecal isolates to ferment protein, peptides and amino acids was examined, a relatively small percentage of these were able to do so and most of these fermented carbohydrates in addition to the amino acid sources provided. The predominant amino acid fermenters were most closely related to *C. coccoides* and *C. botulinum*, but representatives of the *Bacteroides*, *Staphylococcus*, *Enterococcus* and other phylogenetic groups were also found. The results reported here are compared with those obtained from clone libraries prepared from the same environmental samples.

Introduction

Storage of swine manure is associated with the generation of odours. These odours are produced as a result of anaerobic degradation of materials present in manure and include sulphides, organic acids, ammonia, phenols, amines and other volatile compounds (Zahn *et al.*, 1997). Because odour emission from livestock creates a nuisance and therefore may be regulated, there is considerable interest in devising methods to control these emissions. Although a microbiological process, little is known about the types of microorganisms present in stored manure. Previous work in this area has focused primarily on the fate of selected pathogenic organisms in stored and composted manures (Millner and Karns, 2002). More recently, some effort has been made to

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describe the microbial populations associated with swine, but these concentrated on the identification of organisms present in the gastrointestinal tract (Pryde *et al*., 1999; Leser *et al*., 2000; 2002). Much less is known about the predominate culturable microbes present in pit stored manure, an increasingly used form of manure storage.

Because of the environmental characteristics of manure storage, it is assumed that the most numerous species of microorganisms present would be anaerobes. Because cultivation, isolation, and enumeration of strict anaerobes can be tedious, time consuming, and requires some specialized apparatus, few have been willing to conduct a survey of the microbial community in swine manure. In addition, the use of cultivation based methods to analyse microbial communities has been criticized for its failure to isolate a significant portion of the microorganisms present. Indeed it has been estimated that for some environments the use of such methods may only identify as little as one percent of the organisms present (Pace, 1996). The application of non-cultivation based methods of examination of microbial communities; for example, PCR amplification of rRNA genes and sequence based identification of resident microorganisms, offers an attractive alternative to overcome these limitations. However, these methods do not come without their own limitations including: PCR bias, amplification of genes from dead organisms, and variation in the number of rRNA genes with species (Gobel, 1995; Wilson, 1997; Olive and Bean, 1999; Frostegard *et al*., 1999). Furthermore, though much can be learned about the diversity of microorganisms present from the analysis of clone libraries, this sort of analysis tells us little about the phenotype of the organisms present. Cultivation is still required to assemble a collection of organisms to conduct biochemical, genetic, and physiological experiments. The objective of the current study was to conduct an initial survey of microbes present in faeces and stored manure and compare these results to those obtained using PCR-based, direct sequencing of clone libraries from the same environment (Whitehead and Cotta, 2001). The information gathered will be useful in directing future examinations of the ecology of manure storage.

Results

Sampling and enumeration of faecal and stored manure slurry microorganisms

Faecal and manure (slurry) samples were collected from a swine farm and transported back to the laboratory for analysis. Analysis of selected environmental parameters on slurry samples showed they contained approximately 300 mM volatile fatty acids (VFA; the sum of the individual organic acids, acetic, propionic, isobutyric and butyric acids comprised 95% of this mixture); 300 mM ammonia; were 24°C and pH 6.8. Slurry samples were taken at depths of 6 ft (bottom of pit) and 3 ft (approximately at mid-depth). These samples were similar in appearance except the samples from the bottom of the pit contained more visible particulate material.

Microorganisms present in the samples were enumerated by direct microscopic examination as well as by viable plate counts on non-selective, habitat simulating media. Plates were incubated at 25°C (RT) for slurry samples and 37°C for faecal samples and incubated anaerobically for up to 3 weeks. The results are shown in Table 1. The viable counts for LB-carbohydrate plates incubated aerobically were several orders of magnitude lower (approximately 1×10^6, not shown).

In general, viable counts of slurry averaged about 20% of direct counts whereas faecal were about 5% of direct counts. Microscopic examination of samples revealed no noticeable eukaryotes (fungi; protozoa) which is in agreement with previous work in this laboratory where direct amplification of 18 s rRNA genes failed to yield a product (data not shown). Habitat simulating media, containing 40% substrate depleted rumen fluid for enumeration of faecal organisms, and 80% clarified slurry for manure storage organisms yielded the highest viable counts. Use of slurry media for faecal organisms and rumen fluid for manure pit organisms yielded significantly lower plate counts. Faecal and slurry samples were also plated onto the appropriate medium containing the antibiotics tetracycline, erythromycin and tylosin (10 μg ml^{-1}, individually). The proportional counts of organisms capable of growing in antibiotic containing media ranged from approximately 4% for erythromycin in the 6 ft manure slurry sample to

Table 1. Direct and culture counts of bacteria present in faeces (g^{-1}) and swine manure storage pits ml^{-1}.

		Viable counts: medium				
Sample	Direct counts	Rumen fluid medium	Swine slurry medium	Tetracycline[a]	Erythromycin[a]	Tylosin[a]
Faeces	2.0×10^{11}	1.1×10^{10} ($\pm 0.7 \times 10^{10}$)	0.9×10^{9} ($\pm 0.4 \times 10^{9}$)	1.9×10^{9} ($\pm 0.4 \times 10^{9}$)	3.1×10^{9} ($\pm 0.7 \times 10^{9}$)	2.4×10^{9} ($\pm 0.2 \times 10^{9}$)
Pit, 3 ft	1.0×10^{10}	2.8×10^{8} ($\pm 0.8 \times 10^{8}$)	1.0×10^{9} ($\pm 0.3 \times 10^{9}$)	0.5×10^{8} ($\pm 0.1 \times 10^{8}$)	2.1×10^{8} ($\pm 0.3 \times 10^{8}$)	3.2×10^{8} ($\pm 0.5 \times 10^{8}$)
Pit, 6 ft	6.6×10^{9}	0.8×10^{8} ($\pm 0.3 \times 10^{8}$)	1.8×10^{9} ($\pm 1.1 \times 10^{9}$)	1.3×10^{8} ($\pm 0.4 \times 10^{8}$)	0.8×10^{8} ($\pm 0.2 \times 10^{8}$)	2.4×10^{8} ($\pm 0.5 \times 10^{8}$)

a. Antibiotics (10 μg ml^{-1}) were added to either slurry-containing or rumen fluid-containing medium for growth of bacteria in pit and faecal samples respectively.

32% for tylosin in the 3 ft manure slurry sample when compared to enumerations conducted in the absence of added antibiotics. The proportional counts for growth of faecal organisms in these media ranged from 17 to 27% of the total viable counts.

Identification of bacteria present in faeces and manure slurry

One hundred and fifteen colonies randomly selected from the highest dilutions plated were purified and characterized for colony morphology and microscopically for cell morphology. Stocks of pure cultures were maintained on RF agar slants, frozen glycerol stocks and ultimately frozen stock culture suspension (– 80°C). The isolates were identified by 16 s rDNA sequence analysis and comparison to known sequences in GenBank. A dendrogram displaying the phylogenetic grouping of faecal (FPC) and manure pit slurry (PPC) bacteria is shown in Fig. 1. The PPC bacteria obtained from 3 and 6 ft samples seemed to be equally distributed among the phylogenetic groups observed and were treated collectively as manure pit isolates in further analysis and discussion. Both faecal and manure slurry populations were dominated by Gram-positive bacteria with only a few Gram-negative bacteria observed. Most isolates clustered with known relatives, but none displayed complete identity with sequences present in GenBank. Additionally, a few isolates were only distantly related to known species. Strain PPC78 could not be associated with any phylogenetic grouping and only has 70% sequence identity to *Faecalibacterium prausnitzii* (formerly *Fusobacterium prausnitzii*), its closest relative. Strains PPC1 and PPC87 are similar to one another (98% sequence identity), but only distantly related to other Bacteroides-like organisms. Although isolates frequently possessed sequence identities distinct from those of known bacteria, they frequently displayed a high degree of similarity to other manure slurry and faecal bacteria isolated in this study and several OTU's (strains > 99% identity) were noted. For manure slurry bacteria, 52 of 74 sequences (70%) were contained within OTUs, whereas 30 of 41 (73%) of faecal isolates were contained in OTUs.

Results tabulated by Ribosomal Data Project (RDP;) grouping are shown in Table 2. Again, it is apparent that both faecal and manure slurry populations were dominated by Gram-positive organisms, particularly low mol% G + C Gram-positive bacteria (Firmicutes) including various clostridium groups. Faecal isolates tended to be more broadly spread across the RDP groupings, whereas pit organisms were concentrated into fewer groups. The most numerous isolates obtained were members of the *Clostridium botulinum* group for faecal bacteria, and *Clostridium coccoides* and *Enterococcus* groups for pit samples. Isolates with sequence similarity to *Clostridium purinolyticum* were only found in pit samples, whereas a number of groups only had representatives in faecal samples including those most similar to Streptococci and Lactobacilli. The identity of organisms able to grow in the presence of antibiotics commonly used in swine production (although small in number) tended to follow that of the isolates in general indicating no particular group was associated with a particular antibiotic resistance. Interestingly, all organisms originally isolated from tylosin-containing medium were able to grow in the presence of erythromycin when tested later (both are macrolide antibiotics).

Some physiological characteristics

Faecal and pit isolates were further characterized for their ability to grow in various media and to metabolize a variety of sugars and amino acids (Biolog plates). Products of fermentation of glucose were also determined. The percentage of strains displaying a particular characteristic is shown in Table 3.

Nearly all strains were able to grow in synthetic media (i.e. BHI, RGM) without added rumen fluid or clarified slurry employed during their isolation. Of those able to grow in RGM with glucose, approximately one-third were able to grow in medium without added glucose. This is presumably due to growth on the peptone and yeast extract present in this medium. Further investigation of the ability of strains to grow on peptides and amino acids indicated 12% of the pit strains and 21% of the faecal strains displayed increased growth in media containing these substrates over that of unsupplemented RGM (i.e. RGM with no glucose).

The use of the Biolog system to gather phenotypic information on the isolates in this study was useful to describe the major metabolic activities of these populations; however, it provided little definitive data about organisms within a particular group. In general, a high percentage of isolates fermented the carbohydrates examined. The percentage of faecal isolates able to metabolize most carbohydrates was generally higher than for the pit isolates, whereas metabolism of the amino acids tested tended to be higher in pit isolates. Lactic acid was metabolized by some strains. Although no clear metabolic patterns emerged that could be associated with particular phylogenetic groups of organisms, a few general observations could be made (data not shown). With regard to metabolism of carbon and energy sources, isolates within the *C. coccoides* group were among the few that metabolized amino acids, but this varied considerably. For example, strains contained within PPCOTU10 and PPCOTU1 metabolized the amino acids tested but few carbohydrates whereas those in PPCOTU3 gave positive reactions for several carbohydrates but metabolized few

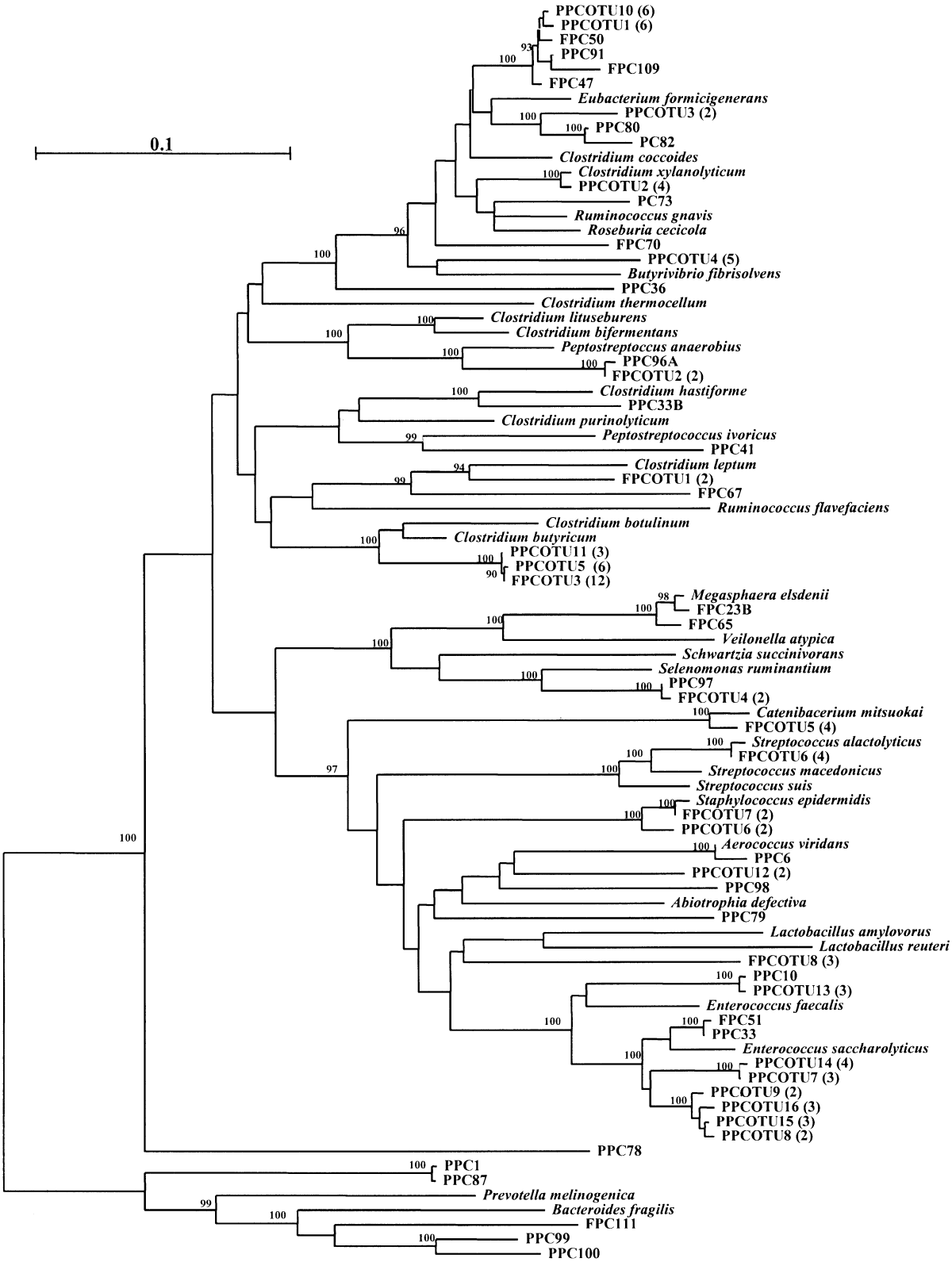

Fig. 1. Phylogenetic analysis of partial eubacterial 16S rDNA gene sequences from swine faeces and stored manure slurry (pit) bacterial isolates. Bar represents 10% sequence divergence. Bootstrap values of 90% or greater, based on 100 replications, are indicated. Coding: F, faecal isolates; P, manure pit isolates. OTU indicates Operational Taxonomical Unit (>99% sequence identity), and the number in parenthesis indicates number of sequences. Bacterial names indicate sequences obtained from GenBank.

Table 2. Phylogenetic grouping of bacteria isolated from swine faecal and manure storage pit samples.

Group[a]	total	faecal	pit	tet[b]	ery[b]	tyl[b]
2.15.1.2 *Bacteroides* group	5	1	4		1	
2.30.3.1 *Sporomusa* group	5	4	1	1		1
2.30.4.1 *C. coccoides* group	32	5	27	9	3	2
2.30.4.5 *C. lituseburense* group	3	2	1	1		
2.30.5 *C. purinolyticum* group	2		2			
2.30.7.12 *Staphylococcus* group	4	2	2		1	
2.30.7.16 *Abiotrophia* group	5		5		2	1
2.30.7.17 *Lactobacilli*	3	3		1	2	
2.30.7.20 *Enterococcus* group	23	1	22	3	1	2
2.30.7.21 *Streptococci*	4	4		1	2	
2.30.8.2 *Acholeplasma-anaeroplasma* group	4	4		1		
2.30.9.1 *C. leptum* group	3	3			1	
2.30.9.2 *C. botulinum* group	21	12	9	2	5	2
Unidentifiable	1		1	1		
Total	115	41	74	20	18	8

a. Group, Ribosome Data Project group number and designation.
b. Bacteria isolated on media containing tetracycline (tet), erythromycin (ery), or tylosin (tyl). The numbers reported here are included in total number isolated and include both faecal and pit bacteria.

of the amino acids tested. Isolates contained within the *C. botulinum* phylogenetic group (e.g. PPCOTUs 11 and 5, and FPCOTU3) exhibited very similar patterns of carbohydrate metabolism with many giving nearly identical positive and negative reactions. Among the *Enterococci*, there was also considerable variability in the reactions observed. Strains in PPCOTUs 7 and 14 yield nearly identical positive reactions for a wide variety of carbohydrate sources while strains in PPCOTUs 8, 9, 11 and 15 were more variable in the patterns of reactions observed. Interestingly, although the Bacteroides-like isolates PPC87, PPC100 and FPC 111 appeared not to be closely related (i.e. displayed less than 90% sequence identity) these strains exhibited nearly identical characteristics when screened for metabolism of Biolog substrates.

The products of fermentation produced during growth in BHI medium were determined. Nearly all strains produced acetic acid, and a large number of strains also produced lactic acid. Propionic and butyric acids were also frequently produced products of fermentation. There appears to be little if any difference between faecal and pit isolates with regard to the types of fermentation products formed. The products of fermentation produced were generally reflected in the products observed in pit samples (i.e. predominantly acetic, propionic and butyric acids). Relatively few strains produced indoles and phenols, compounds often associated with the objectionable odour of swine. Isobutyric and isovaleric acids (products of valine and leucine fermentation respectively) were produced by a number of strains, indicative of amino acid fermentation by those organisms.

Peptide and amino acid fermentation by selected strains of faecal and pit bacteria

Growth of strains that displayed increased growth in peptide and amino acid containing media were studied in greater detail. The ability of these strains to grow on these energy sources as well as glucose and casein was determined. Where possible, strains were grouped by RDP and data averaged. Results are shown in Table 4. Most strains grew best when provided with glucose for growth. Only isolates related to *C. lituseburense* displayed greater growth in peptone and amino acid containing media. Preference for a particular growth substrate ran the full range from those displaying greater growth on peptone while others exhibited greater growth on amino acids. In general, the combination of both substrates yielded greater growth than that observed when supplied separately. Three of these strains, FPC 49 (*C. botulinum*-like), FPC 47 and FPC 50 (both *C. coccoides*-like) produced skatole in BHI medium. Addition of monensin to cultures grown in glucose-containing media inhibited the growth of all strains. Monensin is an ionophore antimicrobial commonly added to cattle feed that is most active against Gram-positive organisms (Russell and Strobel, 1989) and may reduce the fermentation of amino acids in the rumen of these animals.

Discussion

In the current study, microorganisms from swine faeces and deep pit stored manure slurry were isolated, identified by 16 s rDNA sequence analysis, and selected physiological characteristics determined. The results of this examination indicate that the predominant culturable microorganisms from these environments are obligately anaerobic, low mol% G + C Gram-positive bacteria (Firmicutes) who are members of Clostridial, Eubacterial, and *Lactobacillus/Streptococcus* phylogenetic (i.e. RDP) groups. Isolates similar to *Sporomusa* and *Flexibacter/*

Table 3. Some physiological characteristics of faecal and storage pit isolates.

Characteristic	Strains positive (%)		
	Total	Faecal	Pit
Growth in medium:[a]			
BHI	96	100	94
RGM, glucose (0.4%)	96	97	95
RGM without glucose	31	28	33
RGM (no glucose) + peptone, amino acids	16	21	12
Motile	21	2	31
Fermentation of carbohydrates:[b]			
Cellobiose	77	88	74
Fructose	73	90	65
Galactose	67	88	58
Galacturonic acid	59	70	54
Gentiobiose	75	88	69
Glucose	64	80	56
Glycerol	29	25	31
Lactose	57	65	54
Maltose	66	83	58
Maltotriose	65	83	58
Mannitol	43	43	46
Mannose	63	78	56
Raffinose	51	70	43
Rhamnose	38	40	38
Salicin	65	75	60
Sorbitol	47	53	46
Sucrose	50	63	44
Trehalose	56	70	49
d,l Lactic acid	20	13	24
Fermentation of amino acids:[b]			
Alanine	26	15	34
Glutamic acid	11	11	16
Serine	26	13	35
Threonine	15	10	19
Valine	3	5	1
Products of fermentation:[c]			
Acetic acid	97	97	98
Propionic acid	16	26	14
Butyric acid	31	34	29
Isobutyric acid	14	13	14
Caprylic acid	3	8	0
Valeric acid	7	5	8
Isovaleric acid	12	13	11
Caproic acid	14	14	15
Indole	1	2	0
Skatole	2	6	0
Benzoic	1	2	1
4 Methyl valeric acid	4	7	1
Lactic acid	77	81	71

a. Positive growth, $OD_{600} > 0.2$ except for RGM (no glucose) + peptone, amino acids where positive was scored for an increase in $OD_{660} > 0.1$ over RGM without glucose.
b. Reaction on Biolog plates.
c. Growth on BHI medium except when RGM required for growth (1 strain).

Cytophaga/Bacteroides (CFB, Bacteroidetes) groups were also obtained. Although based on relatively few isolates, faecal and manure pit samples exhibited some differences in composition. Pit samples were dominated by organisms similar to *Clostridium coccoides* and *Enterococcus* species, whereas the distribution of species present in faeces appears much broader. Differences in these populations are not completely surprising as environmental and physiological conditions present in these ecosystems are different (e.g. temp, pH) and likely to contribute to selection for different organisms. The most notable difference in the two populations was the large number of *Enterococcus*-like organisms present in pit samples whereas they were nearly absent in faecal samples. Further analysis of these communities will be needed to uncover additional similarities and differences between the microbial populations in these environments.

When compared to the results of previous work in this laboratory on the analysis of 16s rDNA clone libraries (Whitehead and Cotta, 2001), the overall pattern of organisms detected appears similar; that is predominantly low percentage G + C Gram-positive organisms (Firmicutes) with some Sporomusa and CFB (Bacteroidetes) types present. However, in contrast to analysis of the clone libraries, where approximately half of those sequences identified exhibited less than 95% identity with sequences present in GenBank, all but one of the pure culture isolates exhibited >95% sequence identity to members of specific RDP groups. A direct comparison of 16 s rDNA sequences obtained from pure culture isolates to those obtained from the PCR clone library for the same samples yielded few close matches. In fact, of 78 clone sequences analysed only eight displayed greater than 95% identity to pure culture isolates. Four clone sequences had 98% or greater identity to FPCOTU6 of this study which are similar to *Streptococcus bovis*, *S. suis* and *S. alactolyticus*. Other examples of similarities included a clone with 97% identity to FPC23B (*Megasphaera* sp. relative) and two clone sequences greater than 98% identity to FPC100 (*Bacteroiodes* sp. relative). This may indicate that a considerable proportion of the uncultured organisms present represent previously unknown organisms. In addition, whereas most of the pure cultures could be assigned to known RDP groupings, few could be identified as known species further indicating undescribed diversity in these ecosystems. Work is currently underway in this laboratory and in collaboration with others to more completely characterize and identify these new species.

The results of this study are similar to those described by others for examination of clone libraries and for pure cultures isolated from the swine gastrointestinal tract and faeces (Pryde *et al*., 1999; Leser *et al*., 2002). In only one other study has the population present in deep pit stored manure been examined. Spoelstra (1978) conducted a preliminary examination of the microorganisms present in stored manure and noted the presence organisms tentatively identified as *Peptococcus*, *Ruminococcus, Peptostreptococcus* and *Bacteroides* species. Isolates from the current study contain representatives of the phylogenetic groups that these previous isolates likely would be aligned with. To our knowledge, no other attempt has been made to identify these organisms.

Table 4. Growth of peptone and amino acid fermenting strains.[a]

RDP Group	*Abiotrophia*	*Clostridium coccoides*	*Clostridium botulinum*	*Clostridium lituseburen*	*Clostridium sepurinolyticum*	*Enterococcus*	*Staphylococcus*
#, location[b]	1P	3F, 1P	2F, 1P	2F	1P	1P	1F
Growth on RGM plus:							
no addition	0.20	0.33	0.17	0.20	0.21	0.09	0.30
0.4% Glucose	0.99	1.14	0.81	0.35	0.95	0.70	1.02
1% Peptone	0.23	0.43	0.28	0.42	0.23	0.28	0.27
1% Casamino acids	0.24	0.56	0.33	0.62	0.23	0.33	0.20
1% Peptone and 1% Casamino acids	0.48	0.69	0.40	0.87	0.48	0.38	0.27
1% Casein	0.19	0.26	0.13	0.19	0.31	0.22	0.13
Glucose and monensin (% inhibition)	80	91	91	91	80	69	86

a. Growth, OD_{660}. Standard deviations, varied from 0 to 50% across strains, within strain SD generally small.
b. Location: P, pit; F, faeces.

Examination of some growth and physiological characteristics of faecal and pit isolates showed these to be primarily carbohydrate fermenters, although some were able to ferment lactate and amino acids. This is analogous to the rumen which is dominated by saccharolytic organisms (Hespell *et al*., 1998). This is not completely surprising because, as for the rumen, the major energy source available for growth is in the form of plant cell wall polysaccharides (Iannotti *et al*., 1979; Van Soest, 1982; Hobson and Wheatley, 1993). Also similar to ruminal bacteria and other gastrointestinal anaerobes, short-chain volatile fatty acids (e.g. acetic, propionic, butyric) were the major fermentation end products formed by faecal and manure slurry isolates. Although these major fermentation acids contribute significantly to odour because of their high level of production and concentration in manure storage, some of the more unique and noxious compounds associated with swine odour are the result of fermentation of amino acids. Because the fermentation of amino acids can give rise to indoles, phenols, ammonia and amines, we examined the ability of pit and faecal isolates to ferment protein, peptides and amino acids. We found that a relatively small percentage of these were able to do this and most of these fermented carbohydrates in addition to the amino acid sources provided. The predominant amino acid fermenters were most closely related to *C. coccoides* and *C. botulinum*, but representatives of the *Staphylococcus*, *Enterococcus* and other phylogenetic groups were also found. We were able to isolate some strains which were able to grow on peptides and amino acids as energy sources, but in only one case did an isolate grow better on these substrates than carbohydrates. Perhaps, as discovered for the rumen, a population of amino acid fermenting, hyper-ammonia producing bacteria are present at lower dilutions than may be responsible for the extensive protein fermentation that occurs in manure (Russell *et al*., 1988). This fermentation of protein and amino acids is of practical importance in stored manure as many of the compounds associated with swine odour (e.g. indoles and phenols) can be related to degradation of those substrates. Selective isolation procedures might be required to obtain isolates for identification and further study. Interestingly, monensin was strongly inhibitory to the growth of these isolates. Monensin is an antimicrobial that is most effective at inhibiting Gram-positive organisms (Russell and Strobel, 1989). Similar approaches using other compounds might also be employed to reduce selected activities of microbes present. Work is underway here and in other laboratories (e.g. Varel, 1997; Varel and Miller, 2001) to discover methods to control the metabolic activities of bacteria in manure and reduce the production of odorous chemicals.

Experimental procedures

Swine manure pit and faeces sampling

A swine facility near Peoria, IL, was used as the source of swine faecal and manure pit (slurry) samples. Fresh faeces (20–50 g) were recovered from feeder pigs (approximately 100 lbs) fed a corn-soybean based diet. Samples (50–100 ml) from under barn manure storage pits were collected at 3 ft and 6 ft (bottom of pit) depths using a Tank Sampler (NASCO, Fort Atkinson, WI) and transferred to Whirl-Pak sampling bags (NASCO, Fort Atkinson, WI). Samples were kept on ice while returning to the laboratory (approximately 30 min). Faecal samples (1 g) were added to 9 ml of sterile, anaerobic phosphate-buffered saline (0.15 M NaCl, 0.07 M Na phosphate, pH 7.0) and suspended by vortexing. Serial dilutions were performed on this suspension as described below. Microorganisms present in the samples were enumerated by direct microscopic examination (Petroff-Hauser counter).

Media and growth conditions

Isolations and enumerations were performed by plating samples that were serially diluted in anaerobic buffer onto habitat simulating media containing either 40% (v/v) substrate depleted rumen fluid (RF medium; Dehority and Grub, 1976; Leedle and Hespell, 1980) or 80% (v/v) clarified swine manure slurry (Slurry medium; 8000 *g*, 20 min, 4°C). The use of rumen fluid in non-selective, habitat simulating media has

proven to be a good alternative to 'faecal extract' as source of unknown growth factors in the isolation, enumeration, and cultivation of swine faecal and gastrointestinal bacteria (Salanitro *et al*., 1977; Allison *et al*., 1979; Butine and Leedle, 1989). Except where noted for growth of aerobic organisms, the media used in these experiments were prepared anaerobically using the method of Hungate as modified by Bryant (1972). The basic media contained macrominerals, microminerals, buffers, reducing agents and other components as in the RGM medium described by Hespell *et al*. (1987) or anaerobic BHI medium described by Whitehead and Flint (1995). No additional volatile fatty acids were added to slurry containing media. Glucose, xylose, cellobiose, maltose, starch (0.05% w/v each) and peptone (0.3% w/v) were provided as complex carbon, nitrogen and energy sources. The antibiotics tylosin (10 $\mu g\ ml^{-1}$), erythromycin (10 $\mu g\ ml^{-1}$), and tetracycline (10 $\mu g\ ml^{-1}$) were included individually in some media to enumerate organisms capable of growth in the presence of these antimicrobial compounds. Plates were incubated anaerobically in a Coy Anaerobic chamber (Coy Laboratories, Ann Arbor, MI) in a 96% carbon dioxide, 4% hydrogen atmosphere. Plates were incubated at room temperature (approximately 24°C) for manure slurry samples and 37°C for faecal samples. Samples were also plated aerobically onto LB medium supplemented with carbohydrates as above. Growth on plates was examined daily for three weeks and numbers of colonies enumerated. Randomly selected colonies from the highest dilutions were transferred to agar slants (40% RF medium) and incubated at room temperature or 37°C for slurry and faecal samples respectively. Cultures were examined microscopically, purified, and characterized for Gram reaction, colony morphology, fermentation products and substrate utilization. Isolates were identified by determining similarity of 16S rDNA gene sequences with known bacteria (GenBank; http://www.ncbi.nlm.nih.gov/BLAST/).

DNA sequencing and phylogenetic analyses

The 16S rDNA gene from pure cultures was isolated by PCR amplification from boiled cells of an overnight culture using the forward primer 5′-GAGAGTTTGAT (C/T)CTGGCTCAG-3′ (*E. coli* 8–28) and universal reverse primer 5′-GAAGGAG GTG(A/T)TCCA(A/G)CCGCA-3′ (*E. coli* 1540–1522) (Paster *et al*., 1998). Conditions for PCR were: 94°C, 45 s, 55°C, 30 s, 72°C, 2 min, 25 cycles. A final extension step of 72°C, 10 min, completed the reactions. An aliquot of PCR products was analysed by gel electrophoresis to confirm the presence of the correct size product. The PCR product was cloned into the plasmid pCR2.1 (Invitrogen, Carlsbad, CA) using the TA Cloning Kit according to manufacturer's instructions. Randomly selected clones were analysed for correct size inserts in the plasmids by PCR using the m13 forward and reverse sequencing primers. Plasmid DNA was purified using the QIAGEN Wizard kits (QIAGEN, Valencia, CA) according to manufacturer's instructions. The 16S rDNA gene inserts were sequenced at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois Biotechnology Center, Urbana, IL, using the m13 forward and reverse primers and the internal reverse primer 5′-GTAGTTACCGCG GCTGCTG-3′ (*E. coli* 518–500), resulting in approximately the first 500 bases of the gene being sequenced on both strands and the last 500 bases on one strand. The first approximately 500 bases of each 16S rDNA gene sequences were analysed using the Lasergene software (DNASTAR, Madison, WI). Similarity analyses were carried out using the ADVANCED BLAST Program of GenBank (NCBI, NIH, Washington, DC). The 500 base pair sequences from the isolates and sequences recovered from GenBank were aligned using the CLUSTALW program (Thompson *et al*., 1994). Phylogenetic dendrograms were prepared by a neighbour-joining method using the TREECON software (Van de Peer, 1994). An Operational Taxonomic Unit (OTU) indicates those sequences that have 99% or greater sequence identity.

Nucleotide sequence accession numbers

The partial 16S sequences have been deposited in the GenBank database under accession numbers AF445197 through AF445308 and AF450009 through AF450011 (NCBI Entrez Popset 17940448).

Physiological characteristics

The growth of isolates in various media including the ability to utilize various peptide and amino acid sources for growth was monitored spectrophotometrically by measuring optical densities of cultures at 660 nm (Spectronic 21, Milton Roy Company, Rochester, NY). The ability of isolates to metabolize a variety of substrates was examined using reagents and apparatus from Biolog (Hayward, CA) according to the manufacturer's instructions.

Analytical methods

Fermentation end products were determined using gas chromatographic and HPLC methods. Gas chromatography was performed on a Hewlett-Packard model 5890 A gas chromatograph with a HP Innowax capillary column (30 m $\times$ 0.32 mm, 0.5 μm film thickness; Agilent Technologies, Willmington, DE) (Miller, 2001). Helium was the carrier, and peaks were detected by flame ionization and identified by comparison to the retention times of authentic standard compounds. High performance liquid chromatography (HPLC) of organic acids were performed using Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA) and 0.01 M H_2SO_4 as the solvent. Peaks were detected by Waters Model 410 differential refractometer (Waters, Milford, MA) and identified by comparison to retention times of authentic standards. Ammonia was determined using the method of Chaney and Marbach (1962) as modified by Cotta and Russell (1981).

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